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Fluorescence polarization assay: Diagnostic evaluation for porcine brucellosis

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ABSTRACT

Brucellosis in pigs, caused by the bacterium *Brucella suis,* is an important zoonotic infection. In the present study, fluorescence polarization assay (FPA) was standardized and compared with indirect enzyme linked immunosorbent assay (iELISA) and competitive ELISA (cELISA) for diagnosis of porcine brucellosis. Test performances were evaluated using representative panel ($n = 100$), samples from swine brucellosis outbreak ($n = 300$), samples from brucellosis suspected animals ($n = 291$) and sera samples from apparently healthy animals ($n = 1121$). With panel samples, the FPA cut-off ≥11ΔmP was arrived with sensitivity (Se) and specificity (Sp) of 95.00 and 98.75%, respectively. Testing of samples from swine brucellosis outbreak, the diagnostic Se and Sp of 100 and 95.14% by iELISA, 73.91 and 100% by cELISA and 86.96 and 100% by FPA, respectively were recorded. Similarly, in case of swine brucellosis suspected samples, relative performance of FPA with cELISA had revealed higher kappa value of 0.864 with an accuracy of 93.47. Indirect ELISA was found to be highly sensitive but showed cross reactivity mainly for *Yersinia enterocolitica* O9 antibodies than cELISA and FPA. The high specificity of FPA test recorded in various types of samples in the study indicated that, FPA could serve as confirmatory test for individual animal diagnosis, outbreak confirmation, surveillance and quarantine of swine brucellosis cases.

1. Introduction

Brucellosis is one of the most common zoonoses, which affects multiple species leading to reproductive problems in livestock. In swine, brucellosis is caused by *Brucella suis*, and rarely by *B. abortus* and *B. melitensis* (Stoffregen et al., 2007; Olsen et al., 2012; OIE, 2012). The clinical signs noticed are abortion at early or any time of gestation, infertility, orchitis, inflammatory lesions in the joints, reproductive and other organs, paralysis of posterior limbs and lameness (OIE, 2012). *B. suis* has a widespread distribution in both domestic livestock and wildlife population throughout the globe and organism has been isolated from cattle, sheep, dogs, horses, European hares, opossums, armadillos, reindeer, and blue sheep (Olsen et al., 2017). Brucellosis spreads during copulation or by direct contact with aborted fetus within the herds. Although the disease is associated with reproductive losses in swine worldwide, its primary concern is related to its zoonotic capability of causing clinical illness in humans. Addressing brucellosis in livestock reservoir is the most efficient and economical approach for reducing human infections (Olsen et al., 2017).

Diagnosis of swine brucellosis can be achieved by isolation, serological tests and molecular techniques like polymerase chain reaction (PCR). Isolation is the most accurate and confirmatory diagnosis but not

Abbreviations: AUC, area under the curve; cELISA, competitive enzyme linked immunosorbent assay; CI, confidence interval; FN, false negative; FP, false positive; FPA, fluorescence polarization assay; iELISA, indirect enzyme linked immunosorbent assay; mP, milli polarization; NPV, negative predictive value; OPS, o-polysaccharide; PI, performance index; PPV, positive predictive value; RBPT, Rose Bengal plate test; ROC, receivers operating characteristic; Se, sensitivity; Sp, specificity; TN, true negative; TP, true positive; +LR, positive likelihood ratio; -LR, negative likelihood ratio

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suitable for processing large number of samples because of tedious protocols, requirement of bio-containment facility, risk to laboratory personnel and less sensitivity. Molecular methods are reported with reduced sensitivity due to associated inhibitors in DNA samples and also the high cost restricts its use in surveillance studies (Yu and Nielsen, 2010; Wang et al., 2014). Serological tests are commonly used for the diagnosis of porcine brucellosis which includes Rose Bengal plate test (RBPT), indirect and competitive ELISAs (iELISA and cELISA), complement fixation test (CFT) and fluorescent polarization assay (FPA). Most of these serological tests utilize the o-polysaccharide (OPS) antigen of smooth *Brucella* strains. The OPS antigen moiety of *Brucella* is similar to many Gram negative bacteria especially, *Yersinia enterocolitica* O9 and *Escherichia coli* O157. Hence, many of the tests have reduced specificity due to the false positive serological reactions. The tests such as FPA or cELISA have been reported to eliminate cross reactivity associated with *Y. enterocolitica* O9 and *E. coli* O157 (OIE,2012). FPA has been recommended by OIE as screening and confirmatory test which can be used as an additional testing procedure for brucellosis in reference laboratories (OIE, 2012). There are several reports on the evaluation of FPA in different livestock species (Trangadia et al., 2012; Nicola et al., 2010; Schumaker et al., 2010), however, reports on evaluation of FPA for swine brucellosis are scanty. Most of the previous studies were performed using the distinct *Brucella* positive confirmed by isolation of the organism and negative serum samples (Nielson et al., 1999), and evaluation of the assay under field conditions are limited (Paulo et al., 2000).

In India, brucellosis is endemic throughout the country (Upadhyay et al., 2007; Islam et al., 2013; Shome et al., 2014). However, there are meager reports on brucellosis in pigs, although, India ranks 5th pig populous country in the world (DAHD, 2014). Few varying seroprevalence of porcine brucellosis reports are available based on RBPT and iELISA (Nath et al., 2009; Shome et al., 2016; Jindal et al., 2017). The objective of the present study was to standardize FPA for porcine brucellosis and evaluate its efficacy employing the samples collected from the different field conditions such as outbreak, brucellosis suspected farms and stratified random samples to represent the true field conditions.

2. Materials and methods

2.1. Ethics statement

The study was approved by Institutional Animal Ethics Committee, Indian Council for Agricultural Research-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR- NIVEDI), Bengaluru, India. The authors have taken permission from farm owners to publish the data. The work was carried out in biosafety level-II plus laboratory facility.

2.2. Sample source and layout

2.2.1. Representative serum panel

A total of 536 pig serum samples were sourced from 5 organized farms from Karnataka, India during 2015–2016. The samples were categorized as brucellosis seropositive and seronegative by RBPT and iELISA. A panel of 100 representative serum samples consisting of 20 positives and 80 negatives were segregated. Positive serum samples were selected from infected herds with clinical symptoms of brucellosis and confirmed as positive by RBPT, iELISA, *Brucella* genus specific *bcsp* 31 PCR (Baily et al., 1992) and isolation of *B. suis* (Shome et al., 2016). Similarly, negative serum samples were from healthy herds free of brucellosis confirmed by RBPT, iELISA and PCR.

2.2.2. Samples from Brucellosis outbreak

An organized pig farm experiencing brucellosis outbreak in Southern India (Karnataka), confirmed by isolation of the organism and clinical signs such as abortions in later stage of pregnancy, still births, hind leg lameness due to swollen knee joints, orchitis and enlarged lymph nodes was visited and 300 serum samples were collected from both healthy and infected pigs for assay evaluation.

2.2.3. Samples from Brucellosis suspected animals

Based on the characteristic clinical signs of brucellosis, 291 serum samples from pigs collected from North Eastern India (11 districts of Nagaland) and sent to our laboratory for confirmation of brucellosis were assessed in the study.

2.2.4. Samples from apparently healthy animals

Random stratified samples ($n = 1121$) sourced from six North-Eastern states of India (Assam-73, Manipur-430, Nagaland-273, Tripura-28, Meghalaya-178 and Mizoram-139) during 2014–2017 through All India Coordinated Research Project on Animal Disease Monitoring and Surveillance project(AICRP-ADMAS) were included in the study.

Besides these, to rule out cross reactivity of the OPS antigen used in FPA, *E. coli* (O157 H7), and 5 *Yersinia entericolitica* (O1 & 2; O3; O5; O8; O9) serotype specific reference sera (Denka Seiken Co, Tokyo, Japan) were tested.

2.3. Sample collection and processing

Approximately 2–3 mL of blood was collected from the ear vein of animals above 6 months of age in vacutainers without anticoagulant (Becton Dickson, UK), serum was separated from clotted blood after 4–6 h by centrifuging at 3000 rpm for 5 min and clear serum was separated and stored at −20 °C until use.

2.4. Preparation of antigen for FPA

For the FPA test, OPS antigen from *B. abortus* S99 was conjugated with Fluorecein Iso thiocyanate (FITC) as per the guidelines of OIE (OIE, 2009). Briefly, the seed culture of *B. abortus* strain S99 procured from National *Brucella* Culture Repository, Indian Council of Agricultural Research -Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, India was confirmed by Gram's staining, biochemical tests, genus specific *bcsp* 31 and species specific Bruce ladder and AMOS PCRs (Baily et al., 1992; Bricker and Halling, 1994; López-Goñi et al., 2008). Bulk production of *B. abortus* strain S99 was carried out by further culturing the pure colonies on Tryptose Soya Agar (TSA) plates and incubated at 37 °C for 72 h. The grown bacterial cultures were harvested into 2.5% phenol saline buffer, vortexed and incubated for 24 h to ensure total inactivation of *Brucella* cells. A loopful cell suspension was inoculated onto the TSA plates and incubated for 72 h at 37 °C to check the viability. The non-viable *Brucella* cell pellets washed twice with 0.1 M PBS were used for extraction of OPS by acid hydrolysis and conjugated to FITC under alkaline conditions as described previously (OIE, 2009). The FITC conjugated OPS was purified by DEAE affinity chromatography and purified fractions were eluted using 0.1 M phosphate buffer. The total fluorescent intensity of each elution was analyzed and stored at 4 °C until use.

2.5. FPA test protocol

One positive, three negative controls (Pourquier positive and negative control, France) and test sera samples ($n = 1812$) were assayed in the study. The controls and test serum samples were diluted 1:50 by adding 20 μL of each serum into 1 mL of 0·01 M sodium phosphate buffer, pH 7·4 containing 0·15 M NaCl, 0·1% sodium azide and 0·05% lithium dodecyl sulfate in 10 × 75 mm borosilicate tubes. A serum blank measurement was obtained using Fluorescence Polarization Analyser Sentry 200 (Diachemix, USA). The diluted tracer (15 μL) with total fluorescent intensity 2.5-3 × 10^5 was added to each tube,

incubated at room temperature for 3–5 min and second measurement was obtained. The milli polarization values (mP) were automatically calculated by FP analyser by subtracting the initial from the final reading. Positive and negative control sera were included after every 50 samples tested. Data expressed as milli polarization units (mP) were indicative of the amount of antibody present in the serum sample. Delta mP (ΔmP) of each sample (the difference between the calculated mP and average of negative control mP) were obtained as previously described (USDA APHIS report, n.d). The ΔmP was compared with the serological status of the representative panel to obtain a threshold or cut-off ΔmP value with a high specificity and sensitivity using receivers operating characteristic curve (ROC).

2.6. Other serological tests

The serum samples were tested by RBPT as described previously (Alton et al., 1988) and for the RBPT, *B. abortus* S99 colored antigen was procured from Institute of Animal Health and Veterinary Biologicals, Hebbal, Bengaluru, India. Indirect ELISA to detect antibodies against *Brucella,* standardized and evaluated previously in the laboratory using smooth lipolysaccharide (sLPS) antigen was used in the study (Shome et al., 2016). Competitive ELISA (Svanovir TM *Brucella*-Ab c-ELISA test) and commercial FPA (*Brucella abortus* antibody test) kits, respectively manufactured by Svanova Biotech, Sweden and Diachemix, USA were performed as per the instructions of the manufacturer.

2.7. Statistical analysis

Statistical analysis for ROC curve, diagnostic sensitivity (Se) and specificity (Sp), area under the curve (AUC), inter-rater kappa agreements were calculated using MedCalc 15.8 software. The kappa values of < 0.20, 0.21–0.40, 0.41–0.60, 0.61–0.80 and 0.81–1.0, indicate the strength of agreement as poor, fair, moderate, good and very good, respectively (McHugh, 2012).

3. Results

In DEAE affinity chromatography, eluted fractions starting from 16 to 45 mL were found optimal tracer for FPA. The tracer dilution of 1:4 in 0·01 M sodium phosphate buffer, pH 7·4, containing 0·15 M NaCl, 0.1% of sodium azide has given a total intensity of 2.5–3 × 10^5.

Upon testing of the representative serum panel, cut-off of ≥11ΔmP was obtained in ROC curve analysis with Se and Sp of 95.00% (75.1–99.9%) and 98.75% (93.2–100.0%), respectively, and AUC of 0.980 with a significant *P* value < .001 (Fig. 1). Different cut-off values which offered 100% Se to 100% Sp and their respective +LR and –LR are given in Table 1.

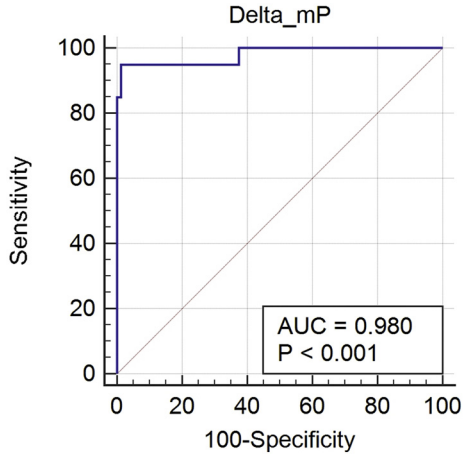

Fig. 1. ROC curve for FPA using representative serum panel.

Table 1
The diagnostic test attributes of FPA at different *Δ mP* cut-off values.

Δ mP cut-off value	Sensitivity (95% CI)	Specificity (95% CI)	+LR	-LR
≥ − 200.99	100.00(83.2–100.0)	0.00(0.0–4.5)	1.00	–
≥ − 0.67	100.00(83.2–100.0)	62.50(51.0–73.1)	2.67	0.00
≥ − 0.36	95.00(75.1–99.9)	62.50(51.0–73.1)	2.53	0.080
≥11.09*	95.00(75.1 - 99.9)	98.75(93.2–100.0)	76.00	0.051
≥15.6	85.00(62.1–96.8)	98.75(93.2–100.0)	68.00	0.15
≥17.52	85.00(62.1–96.8)	100.00(95.5–100.0)	–	0.15
≥196.29	0.00 (0.0–16.8)	100.00(95.5–100.0)	–	1.00

* Selected cut-off with highest Se and Sp.

With the cross reactive serum samples tested, *E. coli* (O157 H7) and four *Y. enterocolitica* antisera (O1 & 2; O3; O5; O8) were detected negative in FPA and cELISA. Whereas, *Y. enterocolitica* O9 antisera had shown 19ΔmP (8mP above the cut-off value) in FPA and in cELISA, 37% reactivity relative to conjugate control (7% above the cut-off) was observed. In iELISA, *Y. enterocolitica* O9 and *E. coli* (O157 H7) antisera have reacted 89% and 68% relative to positive controls, respectively (39% and 18% above the cut-off).

In samples collected from brucellosis outbreak, brucellosis seropositive status was declared by at least two serological tests. Comparison of test results showed the highest performance index (PI) of 195.14 and 186.96 for iELISA and the standardized FPA, respectively. In iELISA, 100% Se and 95.14% Sp and in FPA, 100% Sp with a reduced diagnostic Se of 86.96% were recorded. The diagnostic Se and Sp of individual tests, with PI, PPV, NPV, accuracy and kappa values are presented in Table 2.

Performance of the FPA test was compared with other serological tests employing samples from brucellosis outbreak and results are tabulated in Table 3. The relative diagnostic Se ranging from 75.00–92.94%, Sp from 90.23–96.02% and kappa values between 0.731 and 0.847 were obtained. Among the tests, least Se with higher Sp was recorded for FPA in comparison to iELISA and the highest Se of 92.94 and Sp of 90.23 in comparison to cELISA. Commercial FPA kit results based on positive cut-off provided by the manufacturer were compared with standardized FPA and accuracy/agreement of 93.33% with the highest PI was observed for commercial FPA kit, followed by cELISA, iELISA and least by RBPT.

With brucellosis suspected samples, seroprevalences recorded by iELISA, cELISA and FPA were 64.6%, 62.19% and 57.73%, respectively. Indirect ELISA had the highest Se followed by cELISA and FPA (Table 4). Relative performance of FPA with cELISA had revealed higher kappa value of 0.864 with an accuracy of 93.47 conversely, a reduced kappa of 0.755 with an accuracy of 88.32 were recorded relative to iELISA (Table 5).

An overall seroprevalence of 0.45% was recorded among samples collected from apparently healthy animals sourced from six North Eastern states of India. Anti-*Brucella* antibodies were recorded in the samples from the state of Assam (2.74%) while, samples collected from Tripura, Meghalaya and Mizoram were negative for anti-*Brucella* antibodies (Table 6).

4. Discussion

According to 19th Livestock census of India, pig population is estimated to be 10.29 million and ranks fifth in the world (DAHD, 2014). In India, porcine brucellosis accounts for a median economic loss of US$ 7.1 million annually (Singh et al., 2015) and endemic in the country. Reported studies of porcine brucellosis in India are based on RBPT and iELISA (Nath et al., 2009; Shome et al., 2016) and FPA has not been attempted for diagnosis of swine brucellosis in the country.

In the present study, FPA was standardized for the diagnosis of porcine brucellosis with cut-off ≥11ΔmP and AUC 0.98and with this criteria, 98% animals could be correctly detected. The accuracy of 0.98

Table 2
Performances of various serological tests employing samples collected from swine brucellosis outbreak.

Test	TP	TN	FP	FN	Se (95% CI)	Sp (95% CI)	Performance index	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)	Kappa (95% CI)
RBPT	98	180	5	17	85.22 (77.39–91.15)	97.30 (93.81–99.12)	182.52	95.15 (89.16–97.90)	91.37 (87.22–94.26)	92.67 (89.11–95.35)	0.841 (0.778–0.905)
iELISA	115	176	9	0	100.00 (96.84–100.00)	95.14 (90.97–97.75)	195.14	92.74 (87.11–96.03)	100.00	97.00 (94.38–98.62)	0.937 (0.897–0.977)
cELISA	85	185	0	30	73.91 (64.90–81.66)	100.00 (98.03–100.00)	173.91	100.00	86.05 (81.93–89.35)	90.00 (86.03–93.15)	0.777 (0.703–0.851)
FPA commercial kit (≥10ΔmP)	94	185	0	21	81.74 (73.45–88.33)	100.00 (98.03–100.00)	181.74	100.00	89.81 (85.68–92.84)	93.00 (89.50–95.61)	0.847 (0.794–0.898)
FPA commercial kit (≥8ΔmP)	97	185	0	18	84.35 (76.40–90.45)	100.00 (98.03–100.00)	184.35	100.00	91.13 (87.05–94.02)	94.00 (90.68–96.41)	0.869 (0.811–0.927)
FPA in-house	100	185	0	15	86.96 (79.40–92.51)	100.00 (98.03–100.00)	186.96	100.00	92.50 (88.50–95.19)	95.00 (91.89–97.17)	0.891 (0.838–0.944)

Table 3
Comparison of FPA test with other serological tests employing samples from swine brucellosis outbreak.

Test	TP	TN	FP	FN	Se (95% CI)	Sp (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)	Kappa (95% CI)
RBPT	92	179	8	21	81.42 (73.01–88.11)	95.72 (91.74–98.14)	92.00 (85.30–95.80)	89.50 (85.27–92.62)	90.33 (86.41–93.43)	0.789 (0.716–0.861)
iELISA	93	169	7	31	75.00 (66.43–82.34)	96.02 (91.98–98.39)	93.00 (86.46–96.51)	84.50 (80.05–88.10)	87.33 (83.03–90.88)	0.731 (0.652–0.809)
cELISA	79	194	21	6	92.94 (85.27–97.37)	90.23 (85.46–93.85)	79.00 (71.39–85.01)	97.00 (93.72–98.59)	91.00 (87.18–93.99)	0.789 (0.714–0.864)
FPA commercial kit	87	193	13	7	92.55 (85.26–96.95)	93.69 (89.45–96.60)	87.00 (79.76–91.91)	96.50 (93.11–98.25)	93.33 (89.89–95.88)	0.847 (0.783–0.912)

Table 4
Combinatorial three test results with brucellosis suspected 291 samples from Nagaland.

Number of samples	iELISA	cELISA	FPA
158	+	+	+
6	+	+	−
3	+	−	+
7	−	+	+
0	−	−	+
21	+	−	−
10	−	+	−
86	−	−	−

rather than 1.00 was attributed to reduced diagnostic Se of 95% which was evident at broader 95% CI value 75.1–99.9% (Table 1). For cut-off determination, ΔmP rather than mP has been considered in the study to minimize the temperature effect. Temperature was found with negative linear correlation with mP values in the previous study (Minas et al., 2007). Similar observation was noticed in the present study when a cut-off based on mP has been derived, values around cut-off were varying for certain positive and negative samples when repeated tested. These variations significantly affected the assay Se and Sp. As the temperature raises, free rotation of molecules in a solvent increases and binding affinity decreases which influences the polarization value. Hence, results of each sample were tabulated in relation to negative control values as per USDA APHIS reports (n.d).

The selection of cut-off could be varied according to the need and demand of epidemiological situation. For screening and identification of infected herds, cut-off to obtain maximum Se, while confirmatory diagnosis of individual animal, maximum Sp should be opted. A cut-off with a highest PI (highest Se and Sp) was chosen in the present study, as the assay was intended for both screening and confirmatory diagnosis. The differences in the FPA results were noticed between commercial FPA kit and standardized FPA and this was attributed to non-suitability of cut-off defined by manufacturer in the present context. When the cut-off was reduced to ≥8ΔmP (defined by manufacturer was ≥10ΔmP), there was increased diagnostic Se (2.6%) without affecting the specificity (Table 2). This clearly emphasized the need for regional cut-offs before adopting any diagnostic kit for serosurveillance studies.

The satisfactory diagnostic performance was noticed by RBPT which can be used as screening test for identification of infected herds but while individual animal diagnosis is considered, it suffers from both reduced Se and Sp compare to iELISA and cELISA/FPA respectively. Objective mode of result interpretation associated with RBPT may also contribute significantly for reduced Se and Sp. The standardized FPA exhibited the highest agreements with commercial FPA and cELISA compared to iELISA (Table 3). This might be due to high specificity associated with FPA.

Among the three tests, iELISA had shown the highest PI with reduced Sp, whereas, cELISA and FPA exhibited higher Sp though PI was below iELISA (Table 2). Earlier reports have stated high specificity of FPA and cELISA, while, resolving the problems associated with cross reactive cultures (Nielson et al., 1999; Paulo et al., 2000; OIE, 2012). Similarly, validation of FPA using 201 pig serum samples false positive by conventional tests and iELISA revealed 54% (109) to be positive for brucellosis by FPA (Weiner et al., 2013a). These reports emphasized assay specificity better than other serological tests in resolving false positive serological reactions. In the current study, *Y. enterocolitica* antisera had shown reactivity near cut-off points in both cELISA and FPA suggesting that both tests to certain extent reduced cross reaction due to *Y. enterocolitica* O:9 at field conditions since the hyperimmune antisera used in the study contains higher titer and affinity antibodies than clinical sera.

Table 6
Percent seropositivity of brucellosis in swine populations of North Eastern India using FPA.

State	No. of samples tested	No. of positives	Percent seropositivity (95% CI)
Assam	73	2	2.74 (0.75–9.45)
Manipur	430	2	0.47 (0.13–1.68)
Nagaland	273	1	0.37 (0.0004–2.05)
Tripura	28	0	0 (0–12.06)
Meghalaya	178	0	0 (0–2.11)
Mizoram	139	0	0 (0–2.69)
TOTAL	**1121**	**5**	**0.45 (0.19–1.04)**

Diagnostic performance with serum samples collected from brucellosis outbreak showed the highest PI for iELISA compared to FPA. The diagnostic Sp of 95.14% by iELISA and 100% by both cELISA and FPA were recorded in the present study (Table 2) which were similar to previous reports of 99.9, 99.5 and 98.3% (Paulo et al., 2000) and 98, 97 and 98% (Nielsen et al., 1999). The diagnostic Se of iELISA, cELISA, and FPA in the present study were 100, 73.91 and 86.96% respectively, whereas, in previous studies reported 98.9, 96.6 and 93.8% (Paulo et al., 2000), and 94, 91 and 94% (Nielsen et al., 1999) for iELISA, cELISA and FPA, respectively. Similarity of higher sensitivity of iELISA among the three tests was noticed, whereas, least Se observed in cELISA followed by FPA in the study may be due to difference in sampling method. In the previous reports (Paulo et al., 2000; Nielsen et al., 1999), distinct positive samples were used to draw Se whereas, samples with different infection status, immunoglobulin classes, avidity and concentrations were present in the outbreak farm. This was evident with different Se of FPA noticed in representative panel samples (95.00%) and samples collected from brucellosis outbreak (86.96%). Thus criteria used for selection of positive and negative reference sample are very crucial in evaluating an assay. The evaluation results of Table 1 and Fig. 01 represents samples with clear positive or negative brucellosis status which was evident in PCR positivity, where the organism DNA is detected in serum samples and serology was also positive by RBPT and iELISA. To evaluate a test, these samples serves as unrealistic as in field samples, difference in age, sex, stage of pregnancy and infection status influences the assay performances. Individual tests performance depends on the serological status, according immunoglobulin classes and its present concentration. The test results employing the brucellosis suspected sera samples revealed different sensitivities by cELISA and iELISA. In iELISA alone 7.21% (21) animals were found positive whereas, 3.43% (10) animals were found positive only in cELISA (Table 4). This could be attributed to the different classes of immunoglobulins detected by the tests. Indirect ELISA detects only IgG antibodies irrespective of its competing strength (affinity) with

Table 5
Comparative evaluation of FPA, iELISA and cELISA using samples collected from swine brucellosis suspected cases (n = 291).

Test	TP	TN	FP	FN	Se (95% CI)	Sp (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)	Kappa (95% CI)
iELISA	161	96	7	27	85.64% (79.80–90.32)	93.20% (86.50–97.22)	95.83 (91.0–97.92)	78.05 (71.41–83.50)	88.32 (84.06–91.77)	0.755 (0.678–0.831)
cELISA	165	107	3	16	91.16 (86.04–94.86)	97.27 (92.24–99.43)	98.21 (94.74–99.41)	86.99 (80.71–91.44)	93.47 (89.99–96.02)	0.864 (0.805–0.923)

monoclonal antibodies whereas, cELISA detects antibodies with high competing strength for the binding site irrespective of their immunoglobulin class and subclasses (Ducrotoy et al., 2018).

Although, FPA was reported with high Se of upto 100% in bovines, the present study and the previous studies did not report Se above 95% in pigs (Paulo et al., 2000; Nielsen et al., 1999). This could be due to the utilization of A-dominant OPS antigen extracted from *B. abortus* which varied from the OPS of *B. suis* (Olsen and Tatum, 2017). In earlier studies, serological tests were able to identify 52% of naturally infected feral swine as seropositive (Pedersen et al., 2014) and 17% of culture-positive swine were negative in all serologic tests (Ferris et al., 1995).

When FPA was used for the surveillance of brucellosis using the serum samples collected from North Eastern states of India, the percent prevalence of 2.74 was recorded from Assam (Table 6). Previously, highest prevalence of 87.10% in pigs with a history of abortion (Nath et al., 2009) and lesser than 5% prevalence during 2016 in random sampling survey (Shome et al., 2016) were recorded from Assam. These results, suggests existence of brucellosis in the state with conserved prevalence pattern.

The standardized FPA had been evaluated using two different sampling strategies which indicated two different epidemiological situations; firstly, the assay was evaluated in an organized farm experiencing brucellosis outbreak which was confirmed by isolation (data not shown) and clinical symptoms. Secondly, the purposive sampling from brucellosis suspected animals was carried out. Among both the evaluations, iELISA had shown high diagnostic Se, FPA had revealed the highest diagnostic Sp. The present study revealed iELISA and FPA to be a good combination of tests for confirmatory diagnosis of porcine brucellosis.

Among these two tests, iELISA was better than the FPA and cELISA for the diagnosis of porcine brucellosis. However, the Sp of iELISA was found comparatively less due to cross-reacting antibodies especially *Y. enterocolitica* O9. Competitive ELISA and FPA were reported to posses the ability to differentiate *Y. enterocolitica* O9 infection (Paulo et al., 2000). The standardized FPA also differentiated *Y. enterocolitica* O9 infection. Differentiation of *Y. enterocolitica* O9 is an important factor, since yersinosis commonly occurs in pigs (Weiner et al., 2013b). Considering the complex methodology and high cost of cELISA, FPA was found easily adoptable confirmatory test for brucellosis.

In conclusion, iELISA was found to be more appropriate for screening brucellosis in swine herds, whereas, FPA had advantages in confirmatory diagnosis of individual animal, quarantine of animal before introducing into a new herd. The combination of iELISA and FPA were suitable to obtain highly specific results to prevent unnecessary segregation/removal/depopulation of suspected animals in developing countries like India, where economically lower communities practices swine rearing. The FPA being robust, simpler, inexpensive, adoptable to field conditions had the added advantages to implement into a surveillance programme which could help in the strategic control or eradication of brucellosis to improve pig production.

Conflict of interest statement

We declare that we have no conflicts of interest.

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